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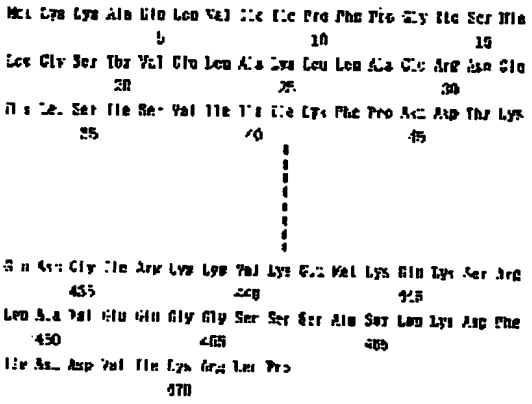
(54) FLAVONOID GLYCOSIDASE GENE

(57)Abstract:
PROBLEM TO BE SOLVED: To obtain a new flavonoid glycosidase gene coding UDP-glucose: flavonoid 3,5-O-glucosyl transferase having a specific amino acid sequence, capable of producing a rose, etc., having blue petals by a petal pigment synthase of Gentiana scabra.
SOLUTION: This flavonoid glycosidase gene is a new UDP-glucose: flavonoid 3,5-O-glucosyl transferase gene coding an amino acid sequence substantially shown by the formula and concerning biosynthesis of a sugar chain structure of gentiodelphin, which is a petal pigment of Gentiana scabra, and the production of a rose having blue petals, etc., is expected by introducing the gene to a plant body according to a conventional method and expressing the gentiodelphin in a race having no method for regulating coloring of a flower color, especially having no blue petal such as the rose. The gene is obtained by extracting mRNA from the petal of the Gentiana scabra, and performing a PCR reaction of a DNA obtained by performing a reverse transcription reaction of the obtained mRNA by using a primer having a specific sequence of „-saccharide transferase.



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* NOTICES *

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CLAIMS

[Claim(s)]
[Claim 1] UDP-glucose which carries out the code of the amino acid sequence substantially shown by the array number 1: Flavonoid 3, a 5-O-glucosyltransferase gene.
[Claim 2] UDP-glucose according to claim 1 originating in a gentian petal: Flavonoid 3, a 5-O-glucosyltransferase gene.
[Claim 3] UDP-glucose according to claim 1 which has the base sequence shown by the array number 2: Flavonoid 3, a 5-O-glucosyltransferase gene.
[Claim 4] claims 1-3 -- UDP-glucose: given in either -- the recombination plasmid containing flavonoid 3 and a 5-O-glucosyltransferase gene.
[Claim 5] The host cell which has a recombination recombination plasmid according to claim 4.
[Claim 6] UDP-glucose which cultivates a host cell according to claim 5 to a culture medium, and is characterized by extracting UDP-glucose:flavonoid 3 and 5-O-glucosyltransferase from a culture: The manufacture approach of flavonoid 3 and 5-O-glucosyltransferase.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to a flavonoid ********** (UFGT) gene. It is related with the enzyme gene which can transfer a glucose to the 3rd place of flavonoid, and the 5th place in more detail.
[0002]
[Description of the Prior Art] Flavonoid is main coloring matter compounds which give various kinds of color tones to vegetation. Various anthocyanin compounds take the lead in a vegetable color tone manifestation in a flavonoid compound, and presenting the color tone of the large range from red to blue is known. An anthocyanin is a glycoside or an acylation glycoside and calls the part except those glycosides an anthocyanidin. The main anthocyanidins are three, BERARUGONIJIN, cyanidin, and delphinidin. The color tone of anthocyanin coloring matter is decided by various factors. There is an inclination to change blue from an orange-red color as the number of the hydroxyl groups of B ring of an anthocyanidin frame is important and the number of hydroxyl groups increases especially. In almost all cases, about the anthocyanin of the petal which presents blue, it is delphinidin. Moreover, the color tone of an anthocyanin is influenced [remarkable] with pH of a solution, the metal ion which lives together, other compounds, etc.
[0003] On the other hand, when the acyl group is aromatic series system organic acids, such as p-coumaric acid and caffeic acid, in an acylation glycoside, it is known that the coloration of a blue system is shown. gene thio delphin which is the anthocyanin of a gentian [-- Goto and T. et al. Tetr.Letters 23 : (36) 3695-3698] (1982) PURACHIKONIN which is the anthocyanin of a platycodi radix [Goto, T. et al. Tetr.Letters 24 : (21) 2181-2184] (1983) The acylation anthocyanin represented It has the structure which caffeic acid combined with intramolecular, and blue is presented to stability also in a neutral region from pH acescence by which an anthocyanin is generally made unstable.
[0004] A glucose combines at least with the 3rd place of a delphinidin frame, the 5th place, and 3' the gene thio delphin which is blue ANTOANIN of a gentian, and at least 3' has the sugar chain structure which caffeic acid combined through the glucose in the 5th place. Therefore, if the enzyme gene which participates in the biosynthesis of the sugar chain structure of the gene thio delphin which is petal coloring matter of a gentian can be isolated, possibility that a flower color can be operated in a blue system is expected by introducing this gene into other plant bodies. Although the enzyme gene which transfers sugar, such as a glucose, to the 3rd place of an anthocyanin until now is known [Ralston and E.J.et al. Genetics 119: 185-197 (1988), Wise, R.P.et al. Plant Mol.Biol.14 : 277-279] (1990) and the enzyme gene which transfers sugar to the 5th place are not known.
[0005]
[Problem(s) to be Solved by the Invention] The technical problem of this invention is to offer the glycosyltransferase gene which may ****** the 2nd place, the 3rd place and the 5th place, among gene thio delphin biosynthesis genes.
[0006]

[Means for Solving the Problem] As a result of repeating research wholeheartedly that the above-mentioned technical problem should be solved, this invention person isolated UDP-glucose:flavonoid 3 and a 5-O-glucosyltransferase gene from the petal of a gentian, succeeds in carrying out the sequencing, and came to complete this invention. Namely, UDP-glucose which carries out the code of the amino acid sequence this invention is substantially indicated to be by the array number 1: They are flavonoid 3 and a 5-O-glucosyltransferase gene.
[0007] The above-mentioned UDP-glucose which has the base sequence this invention is indicated to be by the array number 2 again: They are flavonoid 3 and a 5-O-glucosyltransferase gene. Furthermore, UDP-glucose which this invention cultivates the host cell which has the above-mentioned UDP-glucose:flavonoid 3, a recombination plasmid containing a 5-O-glucosyltransferase gene, and this recombination plasmid, and this host cell to a culture medium, and is characterized by extracting UDP-glucose:flavonoid 3 and 5-O-glucosyltransferase from a culture: It is the manufacture approach of flavonoid 3 and 5-O-glucosyltransferase.
[0008] this invention — setting — "— substantial — " — as long as it has the sugar transition enzyme activity to the 3rd place of flavonoid, and the 5th place — an amino acid sequence given in the array number 1 — setting — 1 or two or more amino acid — addition and deletion — you may permute. Hereafter, this invention is explained to a detail.
[0009]
[Embodiment of the Invention]
[1] Extract mRNA from petals, such as an extract of mRNA, and a separation gentian. First, from this organization, the crude extract of all RNA is obtained, and the impurity of protein, polysaccharide, and others is removed from this, and it refines further using adsorption columns, such as an oligo dT cellulose chromatography and a Pori U-sepharose column. It is eluted, poly A (poly A +) chain fractions can be collected, and mRNA can be condensed to altitude by repeating the same purification 2 to 3 times.
[0010] [2] 3'RACE by beta glycosyltransferase gene sequence primer It attracts attention that the anthocyanin glucose transferring enzyme gene which is one of the petal coloring matter gene thio delphin synthetic enzyme genes of the gentian made into the purpose of PCR this invention is a gene of the enzyme which transfers a glucose at least to beta from the structure of gene thio delphin. Then, UDP-glucose of corn and a barley: The gene which has this and a common array can be made into the candidate of the purpose gene from the gene sequence region of homology of known beta-glycosyltransferase gene cluster represented by flavonoid 3-O-glucosyltransferase and the UDP-guru KURONO sill transferase of an animal.
[0011] In this invention, mRNA first extracted from the petal of a gentian is made into a sample, and it is 3'RACE. A three-dash terminal part is amplified in PCR, and the base sequence is determined. As a primer, it can design based on the information on the array of the above-mentioned known beta glycosyltransferase gene cluster. Specifically, it is the amino acid sequence field which is found out in the amino acid sequence of known beta glycosyltransferase and which was saved at altitude, i.e., (Phe Tyr) (Val Ile), (Thr Cys) His(Ala Gly Ser Cys) Gly. Several sorts compounded from the DNA array corresponding to 16 typical amino acid sequences drawn from the array which consists of 6 amino acid are used. So may not be the degeneration (mix) primer of the primer in this case. Moreover, when performing PCR, even if the array of a primer is not necessarily completely in agreement with the base sequence of the gene aiming at acquisition, magnification by the PCR reaction is possible for it.
[0012] Then, with an obligatory means, cloning of the PCR product is carried out and it carries out sequencing. The acquired DNA array is translated into an amino acid sequence, and if it is a DNA fragment with the consensus sequence of the array of the above-mentioned beta transferring enzyme gene cluster, let it be a candidate gene.
[0013] [3] Generally screening of a cDNA library can perform isolation of the isolation candidate gene of beta glycosyltransferase gene cluster. cDNA is prepared by using as mold mRNA obtained by [1], this is included in a plasmid vector, and various recombinant plasmids are obtained. although an independence duplicate is possible host intracellular, and all can be used as a plasmid vector if the stable maintenance of this cDNA can be carried out — concrete — pSPORT-1 (GIBCO BRL shrine make) etc. — it is mentioned.

[0014] Next, a recombinant plasmid is introduced into Escherichia coli and a cDNA library is produced. It is [2] in order to screen the gene which includes the translation field of a candidate gene from a cDNA library. Several sorts of probes are created based on the array by the side of 3' of the candidate gene which carried out sequencing, and hybridization with this performs.
[0015] Moreover, it is 5'RACE in order to isolate the translation field of a candidate gene. PCR may be performed. Specifically, it is [2]. The primer for reverse transcription is produced based on the array of the determined candidate gene, and specific cDNA is compounded. then, the candidate gene which is made to combine support with the five prime end of cDNA, and does not lap with the support with a complementary support primer and a reverse transcription primer — PCR is performed between specific primers. 1500-2000bp After checking being isolation and that carry out cloning and the array of a three-dash terminal is in agreement with a candidate gene for a DNA fragment, the base sequence of a five prime end is determined.
[0016] [4] In order to select the target gene from the selection candidate genes of the purpose gene About the gene fragment isolated by [3], it is PCR. Or a translation field is taken out by restriction enzyme processing, cloning is carried out to an Escherichia coli expression vector system, and the product of a candidate gene is made to discover with Escherichia coli., Then, Escherichia coli is crushed and the existence of glucose transferring enzyme activity is checked by making the obtained soluble fraction into a sample. Measurement of activity measures two sorts of glucose transferring enzyme activity over the 3rd place of flavonoid, and the 5th place, and if activity is detectable, cDNA introduced into Escherichia coli will judge it to be the gene made into the purpose. Setting to this invention, the decision of a base sequence is a dideoxy chain termination method. [Sanger.F, Science, 214, 1205-1210] (1981), etc. can perform.
[0017] [5] UDP-glucose obtained by the transgenics to a host cell, and the manifestation above : flavonoid 3 and a 5-O-glucosyltransferase gene (henceforth, this invention gene) can be introduced into a suitable host cell, and can be made to high-discover. DNA containing this invention gene is specifically inserted and rearranged to the suitable restriction enzyme part of Vector DNA, Body DNA is prepared, and this is introduced into a host cell. Both an eukaryotic cell and a prokaryotic cell can be used that what is necessary is just what can discover the target gene as a host cell. Cells, such as an animal, vegetation, and yeast, are mentioned as an eukaryotic cell, and Escherichia coli, a Bacillus subtilis, an Actinomyces, etc. are mentioned as a prokaryotic cell.
[0018] If the vector DNA incorporating this invention gene can be reproduced at a host cell, what kind of thing may be used, for example, plasmid DNA, Bacteriophage DNA, etc. will be mentioned. as the vector DNA in case a host cell is Escherichia coli — a plasmid pUC18 / pUC19 and pKK 223-3, pGEX-2T, and pGEX-3 — X and pRIT2 (Pharmacia shrine make); — pGEMEX-1 and pGEMEX-2 (Promega shrine make);pMAL-c and pMAL-p (New England Biolabs shrine make) pET15b (Novagen shrine make) etc. — it can use.
[0019] for carrying out the transformation of the host cell for the recombination object DNA — Hanahan Law ["Molecular Cloning, A Laboratory Manual", 2nd Ed., Cold Spring Harbor Laboratory Press, New York] (1989), and Chung ** — approach [Proc.Natl.Acad.Sci., U.S.A., 86, and 2172] (1989) etc. — it can carry out by using. Culture of the transformant obtained as mentioned above is performed according to the culture approach used for production of the polypeptide by the usual transformant.
[0020] As long as a microorganism is the culture medium which contains the carbon source which can carry out utilization, a nitrogen source, mineral, etc., and can cultivate a transformant efficiently, any of a natural medium and a synthetic medium are sufficient as the culture medium which cultivates the transformant using microorganisms, such as Escherichia coli and a yeast fungus, as a host cell. Alcohols, such as organic acids, such as carbohydrates, such as a glucose, fructose, a sucrose, molasses containing these, starch, or starch hydrolysate, an acetic acid, and a propionic acid, ethanol, and propanol, are used that what is necessary is just that in which each microorganism can carry out utilization as a carbon source.
[0021] As a nitrogen source, a peptone, a meat extract, a yeast extract, corn steep liquor, casein hydrolysate, soybean cake and soybean cake hydrolyzate, various fermentation fungus bodies, the digest of those, etc. are used for the ammonium salt of various inorganic acids, such as

ammonia, an ammonium chloride, an ammonium sulfate, ammonium acetate, and ammonium phosphate, or an organic acid, other nitrogen-containing compounds, and a list. As an inorganic substance, the first potassium of phosphoric acid, the second potassium of phosphoric acid, magmesium phosphate, magnesium sulfate, a sodium chloride, a ferrous sulfate, a manganese sulfate, a copper sulfate, a calcium carbonate, etc. are used.
[0022] Culture is performed under aerobic conditions, such as shaking culture or deep part aeration spinner culture. Culture temperature usually has 10-50-degree C preferably good 30-40 degrees C, and culture time amount is usually 2 - 5 hours preferably for 1 to 10 hours. pH is usually held to 7-8 during culture. Adjustment of pH is performed using an inorganic or organic acid, an alkali solution, a urea, a calcium carbonate, ammonia, etc.
[0023] Isolation purification of the purpose enzyme can be performed combining well-known separation actuation from a culture. For example, processing by modifiers and surfactants, such as a urea, sonication, enzyme digestion, a salting-out, a solvent precipitation method and dialysis, centrifugal separation, an ultrafiltration, gel filtration, SDS-PAGE, isoelectric focusing, an ion exchange chromatography, a hydrophobic chromatography, affinity chromatography, reversed phase chromatography, etc. are mentioned. Hereafter, although an example explains this invention concretely, the range of this invention is not limited by these.
[0024]
[Example]
[Example 1]
(1) 20g of petals of the total RNA extract gentian (form: ****) from the petal of a gentian was ground using the pestle mortar under liquid nitrogen existence. 50ml (25 mM sodium citrate, 0.0025% N-lauroyl ZARUKOSHIN acid sodium, 0.5 mM 2-mercaptoethanol, and 4M guanidine thiocyanate) of guanidine isothiocyanate solutions was added to this, and it stirred, melting at a room temperature. Then, a phenol extract, ethanol precipitate, and LiCl precipitate refined, and all about 5.5mg purification RNA was obtained. Next, the poly A (poly A +) chain fraction was condensed for this to altitude using Oligo(dT)-Latex by known technique [experimental-medicine Vol.7, No.17, and 2065-2068 (1989)].
[0025] (2) Magnification of beta glycosyltransferase gene partial array (2-1) Reverse transcription reaction 3'RACE SYSTEM (product made from GIBCO BRL) It carried out by using. mRNA solution 130 mul (10microg mRNA) 10microM adapter primer (GIBCO BRL shrine make) Solution 10 mul In addition, 65 degrees C incubates for 10 minutes, and it is 2 to Hikami. It was left between parts. It is 10xsynthesis buffer 20 to this. mul, 10mM dNTP mix 10microl, 0.1M DTT 20 mul It is 2 at 42 degrees C moreover. It incubated between parts. Next, SuperScript RT 10 mul In addition, the reaction was performed for 30 minutes at 42 degrees C. Then, a sample is moved to Hikami and it is 10microl. RNase H In addition, incubation was performed for 10 minutes at 42 degrees C.
[0026] (2-2) PCR reaction (2-1) Reverse transcription reaction mixture 1microl 1.25 unit AmpriTaq DNA Polymerase (product made from PERKINELMER CETUS), 10xPCR buffer 5 (product made from TAKARA) mul, 10mM dNTP mix 4microl and 10microM universal amplification primer(product made from GIBCO BRL) 1microl, 20microM beta glycosyltransferase specific array primer 2.5microl It is 50microl about a total amount with sterilized water moreover. It carries out and is PCR. It considered as reaction mixture. this — thermal SAIKURA PJ480 using — PCR (for [PCR program :] 94 ** 7 minutes — one cycle; — for 94 ** 1 minute — a part for -> 42, 48, 50 or 55 **, and 1 — between — a part for -> 72 ** and 1 — between — a part for 30 cycles; 72 degrees C and 7 — between — — 1 cycle) It carried out.
[0027] (2-3) Use TA cloning kit made from Invitrogen for cloning to the plasmid DNA of an PCR product, and the PCR product of the sequence analysis above, and it is pCRII. It introduced into the vector and cloning was performed to F shares of INV alpha. Reading of sequence is DNA sequence equipment 473A (PERKIN ELMER ABI shrine make). It carried out by using. Moreover, the analysis of a DNA sequence is DNASIS (the Hitachi software engineering company make) and SeqEd (product made from PERKIN ELMER ABI) (both software performed.).
[0028] The DNA array acquired as a result of sequence analysis was translated into the amino acid sequence, and considered only what is clearly applied to this array as the array of a

candidate gene as compared with the consensus amino acid sequence of beta glycosyltransferase gene shown in drawing 1 . 288 As a result of carrying out sequence analysis of a part of DNA array about the clone of an individual, the clone conjectured to have 53 beta glycosyltransferase gene sequences was obtained. The obtained clone has been classified into 12 sorts from the array.

[0029] (3) Analysis of the initiation field of beta glycosyltransferase gene (3-1) The stop codon was decided from the array by the side of 5'3 of 12 sorts of candidate genes obtained by production (2) of the primer for RACE PCR', and the complementary array of 30bp(s) which contain a stop codon, respectively was made into the PCR primer. moreover, the array corresponding to an PCR primer — the down-stream complementary array of 30bp(s) was immediately made into the reverse transcription primer.

[0030] (3-2) cDNA composition 5'RACE SYSTEM (product made from GIBCO BRL) It carried out by using. mRNA solution 14microl (1microg) 2.5 muM Reverse transcription primer solution 1microl In addition, 70 degrees C incubates for 10 minutes, and it is 1 to Hikami. It was left between parts. They are 10xsynthesis buffer 2.5microl, 10mM dNTP mix 1microl, and 0.1M DTT 2.5 to this. mul, 25mM MgCl2 3microl, reverse transcriptase SuperScript RT 1 mul In addition, it incubated at 70 degrees C continuously for 30 minutes by 50 degrees C for 15 minutes. Then, 1 mul RNase H In addition, it incubated for 10 minutes at 55 degrees C. Compound cDNA 5'RACE SYSTEM (GIBCO BRL shrine make) The attached protocol refined using the attached cartridge column.

[0031] (3-3) Magnification of a DNA fragment including an initiation field (3-2) It is Support DNA (product made from CLONTECH) to obtained cDNA. T-four Ligase It used and was made to join together with a conventional method. cDNA which combined Support DNA — GENE Amp PCR System9600 (product made from PERKIN ELMER) (3-1) be alike — Li and support primer (CLONTECH shrine make), the designed PCR primer — using — PCR (PCR program: — for 94 ** and 45 seconds — a part for ->55-65 degree C, -> 72 during 45 seconds **, and 2 — between — 35 cycles) It carried out.

[0032] (3-4) 5'RACE Cloning to the plasmid DNA of an PCR product, and magnification product of the sequence analysis above (2-3), Cloning was performed similarly. Sequence (2-3), It carried out using the same equipment and the base sequence of the initiation field of 12 candidate gene was determined.

[0033] (4) Isolation of the translation field DNA fragment of a candidate gene (4-1) PCR The restriction enzyme recognition sequence for including in 5' edge of 24 bases (or 23 bases) equivalent to the 2-9th amino acid residue of the amino terminal of the amino acid sequence of the production 12 candidate gene of a primer at a vector and the array which attached three bases of GCG to the 5' edge further were made into the single-sided primer. Another primer was considered as the restriction enzyme recognition sequence for including in 5' edge of 24 bases (or 23 bases) which have the array which does not form complementary structure at a vector, and the array which added three bases of GCG to the 5' edge further with the above-mentioned primer in the complementary array of 3' untranslation region of a candidate gene.

[0034] (4-2) RT-PCR Candidate gene ORF to depend Production 2.5mM dNTP mix 1.6microl of a field DNA fragment, and 20microM PCR Both primers each 2.0 mul mixing — sterilized water — a total amount — 10microl ** — carrying out — Ampli Wax PCR Gems 100 (product made from PERKIN ELMER) adding — thermal SAIKURA PJ9600 (PERKIN ELMER shrine make) 10-minute 80 degrees C and 25 more degrees C, and 5 It part-incubated. To this, 10xUITma buffer (PERKIN ELMER shrine make) 10microl, 25mM MgCl2 6microl and UITma DNA Polymerase 1 (product made from PERKIN ELMER) mul, Reverse transcription product solution 1 produced by (2-1) mul and sterilized water 72microl It adds. Thermal SAIKURA PJ9600 (PERKIN ELMER shrine make) It uses. PCR (PCR program: — a part for 96 degree C and 1 — between — a part for 1 cycle;95 ** and 1 — between — a part for ->55 degree C and 1 — between — a part for -> 72 ** and 1 — between — a part for 35 cycle;72 ** and 7 — between — 1 cycle) It carried out.

[0035] Reaction mixture performed phenol chloroform processing and ethanol precipitate processing, was processed with the restriction enzyme suitable for each primer, and performed phenol chloroform processing and ethanol precipitate processing. Next, 1% Seakem GTG Agarose

(product made from FCM) Electrophoresis was carried out, the detected DNA bands were collected according to the conventional method, and it considered as the DNA fragment built into an expression vector.
[0036] (5) Screening of the purpose gene using an Escherichia coli manifestation system (5-1) Candidate gene ORF Expression vector pET-15b of a field Candidate gene ORF produced by the inclusion above About the DNA fragment of a field, it is Ligation Kit (product made from TAKARA). It uses, includes in expression vector pET15b, and is Escherichia coli JM 109. Cloning was carried out to the stock. DNA C KUNSU performed the check of a recombination object.
[0037] (5-2) Protein manifestation host Escherichia coli BL21 (DE3) of a candidate gene expression vector The candidate gene expression plasmid DNA produced by the introductory above to a stock was isolated, and the transformation was carried out to Escherichia coli BL21 (DE3) stock.
[0038] (5-3) Add the Escherichia coli in which the purpose gene by sugar transition activity detection carried out the screening transformation by LB culture medium, add IPTG (isopropyl thio-beta-GARAKUSHIDO, final concentration 1mM) after shaking culture at 37 degrees C to O.D.=0.6, and it is further 3 at 37 degrees C. Time amount shaking culture was carried out. It is a buffer after a harvest and about a fungus body pellet. [50 mM Pipes-K] (pH7.0) One ml It suspends and they are Triton X-100 5microl and 10mg/ml Lysozyme 1microl 20%. They are freezing (-80 degrees C) after leaving it for 15 minutes at 30 degrees C in addition, and fusion 3 **********. Then, it carried out until suspension depended sonication on transparence, and centrifugal was carried out by 4 ** with the micro centrifuge for 15000rpm and 15 minutes, and the soluble fraction of supernatant liquid was obtained.
[0039] then, a soluble fraction — a demineralization column — carrying out — Hi-Trap desalting (Pharmacia manufacture) — using — buffer [50 mM Pipes-K (pH7.0), 2% Triton X-100, 0.2mM DTT, 1mM PMSF, 50 mug/ml leupeptin, and 0.54TIU aprotinin] 20 ml as pretreatment of a column — 5 ml/min. — a sink — continuing — this fraction — 1ml It desalted by passing. Three moreml It is a buffer A sink and 800 mul Every fractionation is carried out and it is 2. The outflow fraction of eye watch was used for activity detection.
[0040] Soluble fraction 40microl which performed the above-mentioned demineralization 925 KBq/mul [14C]-UDPG 5microl, Substrate coloring matter solution [Del (Delphinidin), Del-3G (Delphinidin 3-O-glucoside), Cya (Cyanidin), 5mM hydrochloric acid which dissolved Cya-3G (Cyanidin 3-O-glucoside) respectively, In control without substrate coloring matter, they are 5mM(s). Hydrochloric-acid] 5 mul In addition, it incubates for 30 minutes at 30 degrees C, and is chloroform:5%. A hydrochloric acid/methanol = it is 50microl about 2:1. In addition, the water layer was isolated preparatively.
[0041] They are an opposition column and muBondapak C18 about a water layer. It dissociates by used HPLC and is 280nm. It acted as the monitor of the absorbance. the sample furthermore isolated preparatively every [during 30 seconds] by the fraction collector — 2.5ml of liquid scintillators In addition, the activity of each fractionation was measured by liquid scintillation counter LS6000TA (made in Beckmann). At the soluble fraction of the clone containing pET15b without an insertion, after checking that the sugar transition reaction to substrate coloring matter does not occur, when the sugar transition activity of the clone containing a candidate gene was investigated, the sugar transition reaction to four sorts of substrates was accepted by one clone in a candidate gene. The reaction result according to substrate is shown in drawing 2 .

[0042] (6) cDNA Isolation of the purpose gene cDNA from a library (6-1) mRNA used as the production sample of the gentian petal cDNA was refined using Quick Pep (Pharmacia manufacture) from the gentian petal. cDNA composition is mRNA 1 mug It uses and is SuperScript Plasmid System for cDNA Synthesis (product made from BRL). It carried out.
[0043] (6-2) cDNA cDNA produced by the production above of a library — 1% SeaKem GTG Agarose (FMC company make) electrophoresis — carrying out — about 1.3 — the field of the die length of -2.5kbp — from migration gel — collecting — SUPREC-01 (TAKARA shrine make) It refined. This DNA Ligation Express (product made from CLONTECH) Ligation is carried out to a plasmid pSPORT1, and it is an ElectroMAX DH10B competent cel (product made from GIBCO

BRL). Gene Pulser (product made from Bio Rad) The transformation was used and carried out.
[0044] (6-3) Screening GENETRAPER cDNA Positive Selection System of the purpose gene cDNA (product made from GIBCO BRL) It carried out by using. The DNA sequence performed the check of the obtained cDNA clone. A probe is 5'-ATGAAGAAAGCAGAGTTGGTTATCA-3'. A duplicate primer is 5'-CATTTCCAGGGATTAGCCATGTTGG-3'. The synthetic DNA with an array was used.
[0045] (6-4) The clone obtained by the sequencing above of the candidate gene cDNA determined a part of DNA arrays, and after checking that it was the clone which is in agreement with the array of the purpose gene, all base sequences were determined (array number 1). Moreover, the amino acid sequence expected from this is shown in the array number 2.
[0046]
[Layout Table]
array number: -- die-length [of one array]: -- mold [of 473 arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide array Met Lys Lys Ala Glu Leu Val Ile Ile Pro Phe Pro Gly Ile Ser His 5 10 15 Leu Gly Ser Thr Val Glu Leu Ala Lys Leu Leu Ala Glu Arg Asn Glu 20 25 30 His Leu Ser Ile Ser Val Ile Ile Ile Lys Phe Pro Asn Asp Thr Lys 35 40 45 Val Ser Asn Leu Leu Lys Ser Leu Ser Thr Ala Ser Arg Ile Lys Val 50 55 60 Ile Glu LeuLys Gln Glu Thr Ile SerIle Glu Thr Gly Pro Leu Phe 65 70 75 80 Ile Gln Lys Phe Ile Glu Ser His Lys Ala Gln Val Arg Asp Phe Leu 85 90 95 Ala Gly Val Ser Ala Cys Glu SerVal Glu Leu Ser Gly Val Val Ile 100 105 110 Asp Met Phe Cys Thr Ser Met IleAsp Val Ala Asn Glu Phe Glu Val 115 120 125 Pro Ser Tyr Val Phe Phe Thr Ser Ser Ala Ala Met Leu Gly Leu Trp 130 135 140 Phe His Phe Gln Ser Leu Arg Asp Asn Phe Gly Lys Tyr Val Asp Ile 145 150 155 160 Lys Asp Ser Glu Thr Val Leu Ser Ile Pro Ala Phe Gln Asn Leu Val 165 170 175 Pro Tyr Gly Val Leu Pro MetPhe Ile Phe Asn Thr Glu Asp Gly Cys 180 185190 Asp Ala Ser Leu Asp Ile Gly LysArg Phe Arg Glu Thr Lys Gly Ile 195 200 205 Ile Ile Asn Thr Phe Leu Glu Leu Glu Ser His Ala Ile Glu Ser Leu 210 215 220 Ser Thr Asp Glu Thr Ile Pro Pro Val Tyr Thr Val Gly Pro Ile Leu 225 230 235 240 Gly Pro Lys Gly Ser Ser Ile Glu Ser Leu Glu Thr Glu Lys Ile Leu 245 250 255 Lys Trp Leu Asp Met Gln Pro GluLys Ser Val Val Phe Leu Cys Phe 260 265 270 Gly Ser Leu Gly His Phe GlyGluAla Gln Val Lys Glu Ile Ala Tyr 275 280 285 Ala Leu Glu Gly Ser Gly His Arg Phe-Leu-Trp-Ser-Leu-Arg-Lys-Pro 290 295 300 Pro Pro Leu Gly Lys Phe Glu Gly Pro-Gly-Glu-Tyr-Glu-Asn Leu Glu 305 310 315 320 Glu Val Leu Pro Glu Gly Phe Leu Glu Arg Thr Ala Asn Thr Gly Met 325 330 335 Val Ile Gly Trp Ala Pro Gln Thr Ala Val Leu Ser His Ser Ala Val 340 345 350 Gly Gly Phe Val Ser His Cys Gly Trp Asn Ser Thr Leu Glu Ser Ile 355 360 365 Trp Phe Gly Val Pro Val Ala Thr Trp Pro Leu Phe Ala Glu Gln Gln 370 375 380 Met Asn Ala Phe Glu Leu Val Lys Glu Leu Gly Leu Ala Val Glu Val 385 390 395 400 Lys Met Asp Tyr Lys Lys Asp Tyr Lys Asn Pro Asp Ala Asp Glu Ile 405 410 415 Val Arg Ala Asp Val Ile Glu Glu Lys Ile Lys Ile Leu Met Asp Pro 420 425 430 Glu Asn Gly Ile Arg Lys Lys Val Lys Glu Met Lys Glu Lys Ser Arg 435 440 445 Leu Ala Val Glu Glu Gly Gly Ser Ser Ser Ala Ser Leu Lys Asp Phe 450 455 465 Ile Asn Asp Val Ile Lys Arg Leu Pro 470 [0047] array number: -- die-length [of two arrays]: -- mold [of 1597 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- class [of straight chain-like array]: -- the cDNA to mRNA origin living thing name: -- class [of gentian cell]: -- notation:CDS existence location: showing the description description of a petal array -- 39..1460 description The determined approach: EAAGAAAGACA AAAAATTGAG CAAAAAGAAG GGGAAAAAAT GAAGAAAGCA GAGTTGGTTA 60 TCATCCCATT TCCAGGGATT AGCCATCTTG GTTCAACAGT TGAATTAGCT AAGCTTCTTG 120CAGAGAGAAA TGAACATCTC TCCATTTCAG TCATCATCAT CAAGTTCCCA AATGATACAA 180AGGTTTCCAA CCTCTTAAAA TCCCTATCCA CAGCTTCACG GATAAAGGTC ATTGAGCTTA 240AACAAGAAAC CATTTCAATT GAAACCGGCC CACTTTTTAT ACAAAAATTC ATCGAATCCC 300ATAAGGCCCA GGTGAGAGAT TTTCTTGCTG GAGTTTCTGC TTGTGAATCT GTTGAACTCT 360CCGGGGTTGT GATAGATATG TTCTGTACCT CCATGATTGA TGTTGCCAAT GAGTTTGAGG 420TTCCAAGCTA TGTGTTTTTC ACATCTAGTG CTGCAATGCT TGGTCTTTGG TTCCATTTTC 480AAAGCCTTAG GGACAATTTC GGTAAATATG TAGATATTAA AGATTCTGAG ACTGTCTTAA 540 GCATCCCTGC TTTCCAAAAT CTTGTTCCTT ATGGAGTATT GCCTATGTTT ATATTCAATA

600CGGAAGATGG ATGTGATGCA-TCTTTAGATA TAGGTAAAAG-ATTTAGGGAG
ACAAAAGGGA 660TCATAATCAA TACATTTCTT-GAGCTTGAAT CCCATGCTAT-
TGAATCTTTG TCCACAGATG 720AAACCATCCC ACCGGTTTAC-ACTGTAGGGC
CAATATTGGG-ACCAAAAGGC AGCAGTATCG 780AAAGCCTGGA AACCGAGAAG-
ATTTTGAAAT GGCTTGATAT-GCAGCCTGAG AAGTCTGTAG 840TTTTCCTTTG
TTTTGGTAGC CTTGGTCATT TTGGTGAGGC CCAAGTGAAG GAAATCGCAT
900ATGCACTAGA GGGTAGTGGC CATAGATTCT TGTGGTCATT AAGAAAGCCA
CCACCTTTAG 960GGAAATTTGA AGGCCCTGGT GAGTATGAGA ACTTGGAAGA
GGTCCTCCCA GAAGGGTTTT 1020TAGAACGCAC AGCTAACACC GGAATGGTGA
TTGGATGGGC CCCACAGACG GCCGTGTTGT 1080CTCACTCAGC TGTGGGAGGT
TTCGTGTCGC ATTGTGGATG GAATTCAACA CTGGAAAGCA 1140TTTGGTTTGG
TGTCCCAGTG GCAACCTGGC CTCTGTTTGC TGAGCAGCAG ATGAATGCAT
1200TTGAATTGGT GAAGGAATTG GGCTTGGCTG TGGAGGTTAA GATGGATTAT
AAAAAAGATT 1260ACAAGAATCC TGACGCGGAC GAAATAGTGA GAGCTGATGT
TATAGAAGAG AAAATCAAGA 1320TATTGATGGA TCCTGAAAAT GGAATCAGAA
AGAAAGTGAA GGAAATGAAA GAAAAGAGCA 1380GGCTGGCTGT TGAGGAAGGA
GGATCATCTT CTGCGTCCCT CAAAGATTTT ATTAATGATG 1440TGATCAAAAG
ACTTCCATGA TTTAGCTCTT CCAAATTGGT TCAATAGATT TTATGGGCAA
1500AACTGTTAAG TTGCTCTTTG CATTAGAGCA AATGCTCTGA TTTAATGTAA
GTTTTTATTT 1560 TTATTTTTTA TTTATCTGAA ATTAAGTTTA CAAAAAA 1597 [0048]
[Effect of the Invention] UDP-glucose which is the enzyme gene which participates in the biosynthesis of the sugar-chain structure of the gene thio delphin which is petal coloring matter of a gentian according to this invention: Flavonoid 3 and a 5-O-glucosyltransferase gene are offered. If the gene of this invention is introduced into a plant body according to a well-known approach, creation of the blue petal by making gene thio delphin discover in a form without blue petals, such as accommodation of the color of a flower color, especially a rose, is expectable.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] The comparison of the amino acid sequence of various beta glycosyltransferase genes is shown.
[Drawing 2] According to substrate of one clone sugar transition enzyme activity was accepted to be [A. The reaction result of Del (Delphinidin), B.Del-3G (Delphinidin 3-O-glucoside), C.Cya (Cyanidin), and D.Cya-3G(Cyanidin 3-0-glucoside)] is shown.

[Translation done.]

全項目

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(54)【発明の名称】フラボノイド配糖化酵素遺伝子
(51)【国際特許分類第6版】

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   C07H 21/04
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         9/10
//(C12N  1/21
   C12R  1:19    )
  (C12N  9/10
   C12R  1:19    )
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【FI】

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   C12N 15/00     ZNA A
   C07H 21/04         B
   C12N  1/21
         9/10
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(57)【要約】
【解決手段】UDP－グルコース:フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子、該遺伝子を含む組換えプラスミド、該組換えプラスミドを有する宿主細胞、ならびに該宿主細胞を培地に培養し、培養物よりUDP－グルコース:フラボノイド3，5－O－グルコシルトランスフェラーゼを採取することを特徴とする、UDP－グルコース:フラボノイド3，5－O－グルコシルトランスフェラーゼの製造方法。
【効果】本発明によれば、リンドウの花弁色素であるゲンチオデルフィンの糖鎖構造の生合成に関与する酵素遺伝子であるUDP－グルコース:フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子が提供される。本発明の遺伝子を公知の方法に従い植物体に導入すれば、花色の色

彩の調節、特にバラ等の青色花弁をもたない品種においてゲンチオデルフィンを発現させることによる青色花弁の創出が期待できる。

【特許請求の範囲】
【請求項1】実質的に配列番号1で示されるアミノ酸配列をコードするUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子。
【請求項2】リンドウ花弁に由来する、請求項1記載のUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子。
【請求項3】配列番号2で示される塩基配列を有する請求項1記載のUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子。
【請求項4】請求項1～3いずれかに記載のUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子を含む組換えプラスミド。
【請求項5】請求項4記載の組み換え組み換えプラスミドを有する宿主細胞。
【請求項6】請求項5記載の宿主細胞を培地に培養し、培養物よりUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼを採取することを特徴とする、UDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼの製造方法。

【発明の詳細な説明】
【0001】
【発明の属する技術分野】本発明は、フラボノイド配糖化酵素（UFGT）遺伝子に関する。さらに詳しくはフラボノイドの3位，5位にグルコースを転移することのできる酵素遺伝子に関する。
【0002】
【従来の技術】フラボノイドは、植物に各種の色調を与える主要な色素化合物である。フラボノイド化合物の中で植物の色調発現の中心となるのが、種々のアントシアニン化合物であり、赤から青までの広い範囲の色調を呈することが知られている。アントシアニンは配糖体もしくはアシル化配糖体であり、それらの配糖体を除いた部分をアントシアニジンと呼ぶ。主なアントシアニジンはペラルゴニジン、シアニジン、デルフィニジンの3つである。アントシアニン色素の色調は種々の要因によって決まる。中でもアントシアニジン骨格のB環の水酸基の数は重要であり、水酸基の数が増すに従って橙赤色から青色に変わる傾向がある。青色を呈する花弁のアントシアニンについてはほとんどの場合デルフィニジンである。また、アントシアニンの色調は溶液のpHや共存する金属イオン、他の化合物などによっても著しい影響を受ける。
【0003】一方、アシル化配糖体において、そのアシル基がp-クマル酸やコーヒー酸などの芳香族系有機酸である場合、青色系の呈色を示すことが知られている。リンドウのアントシアニンであるゲンチオデルフィン [Goto, T. et al. Tetr. Letters 23: (36) 3695-3698 (1982)] や、キキョウのアントシアニンであるプラチコニン [Goto, T. et al. Tetr. Letters 24: (21) 2181-2184 (1983)] に代表されるアシル化アントシアニンは、分子内にコーヒー酸が結合した構造を持ち、一般的にアントシアニンが不安定であるとされるpH弱酸性から中性領域においても安定に青色を呈する。
【0004】リンドウの青色アントアニンであるゲンチオデルフィンは、デルフィニジン骨格の3位，5位，3’位にグルコースが結合し、3’位，5位にはグルコースを介してコーヒー酸が結合した糖鎖構造を持つ。従って、リンドウの花弁色素であるゲンチオデルフィンの糖鎖構造の生合成に関与する酵素遺伝子を単離することができれば、該遺伝子を他の植物体に導入することにより花色を青系に操作できる可能性が期待される。これまでアントシアニンの3位にグルコース等の糖を転移する酵素遺伝子が知られているが [Ralston, E.J. et al. Genetics 119: 185-197 (1988) 、Wise, R.P. et al. Plant Mol. Biol. 14: 277-279 (1990)]、5位に糖を転移する酵素遺伝子は知られていない。
【0005】
【発明が解決しようとする課題】本発明の課題は、ゲンチオデルフィン生合成遺伝子のうち、3位，5位の2位を配糖化しうる糖転移酵素遺伝子を提供することにある。
【0006】
【課題を解決するための手段】本発明者は、上記課題を解決すべく鋭意研究を重ねた結果、リンドウの花弁よりUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子を単

離し、その配列決定をすることに成功し、本発明を完成させるに至った。すなわち、本発明は、実質的に配列番号1で示されるアミノ酸配列をコードするUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子である。
【0007】本発明はまた、配列番号2で示される塩基配列を有する上記UDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子である。さらに、本発明は、上記UDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子を含む組換えプラスミド、該組換えプラスミドを有する宿主細胞、ならびに該宿主細胞を培地に培養し、培養物よりUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼを採取することを特徴とする、UDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼの製造方法である。
【0008】本発明において、「実質的に」とは、フラボノイドの3位，5位への糖転移酵素活性を有する限り、配列番号1に記載のアミノ酸配列において、1もしくは複数のアミノ酸が付加、欠失、置換されていてもよい。以下、本発明を詳細に説明する。
【0009】
【発明の実施の形態】
[1] mRNAの抽出及び分離リンドウ等の花弁よりmRNAを抽出する。まず該組織より全RNAの粗抽出物を得、これよりタンパク質、多糖類、その他の夾雑物を除去し、オリゴdTセルロースクロマトグラフィー、ポリU－セファロースカラムなどの吸着カラムを用いて更に精製する。ポリA（ポリA＋）鎖画分を溶出し集め、同様の精製を2～3回繰り返すことによってmRNAを高度に濃縮することができる。
【0010】[2] „糖転移酵素遺伝子配列プライマーによる3' RACE PCR本発明の目的とするリンドウの花弁色素ゲンチオデルフィン合成酵素遺伝子の一つであるアントシアニングルコース転移酵素遺伝子は、ゲンチオデルフィンの構造より、グルコースを„位に転移する酵素の遺伝子であることが注目される。そこで、例えばトウモロコシとオオムギのUDP－グルコース：フラボノイド3－O－グルコシルトランスフェラーゼや動物のUDP－グルクロノシルトランスフェラーゼに代表される既知の„－糖転移酵素遺伝子群の遺伝子配列相同領域より、これと共通の配列を有する遺伝子を目的遺伝子の候補とすることができる。
【0011】本発明においてはまず、リンドウの花弁より抽出したmRNAを試料とし、3' RACE PCRにて3' 末端部分の増幅を行い、その塩基配列を決定する。プライマーとしては、上記既知の„糖転移酵素遺伝子群の配列の情報を基に設計することができる。具体的には、既知の„糖転移酵素のアミノ酸配列において見いだされる、高度に保存されたアミノ酸配列領域、即ち(Phe Tyr)(Val Ile)(Thr Cys)His(Ala Gly Ser Cys)Gly の6アミノ酸からなる配列から導かれる代表的な16個のアミノ酸配列に対応するDNA配列より合成した数種を用いる。この場合のプライマーはデジェネレート（ミックス）プライマーでも、そうでなくてもよい。また、PCRを行う場合、プライマーの配列は必ずしも獲得を目的とした遺伝子の塩基配列に完全に一致しなくても、PCR反応による増幅が可能である。
【0012】続いてPCR産物を常套的な手段にてクローニングし、配列決定する。得られたDNA配列はアミノ酸配列に翻訳し、上記の„転移酵素遺伝子群の配列の共通配列を持つDNA断片であれば、候補遺伝子とする。
【0013】[3] „糖転移酵素遺伝子群の単離候補遺伝子の単離は、一般的にはcDNAライブラリーのスクリーニングによって行うことができる。[1]で得られたmRNAを鋳型としてcDNAを調製し、これをプラスミドベクターに組み込み、種々の組換え体プラスミドを得る。プラスミドベクターとしては、宿主細胞内で自立複製可能で該cDNAを安定保持できるものであれば、いずれをも用いることができるが、具体的には、pSPORT-1 (GIBCO BRL 社製) 等が挙げられる。
【0014】次に、組換え体プラスミドを大腸菌に導入してcDNAライブラリーを作製する。cDNAライブラリーから候補遺伝子の翻訳領域を含む遺伝子をスクリーニングするには、[2] で配列決定した候補遺伝子の3' 側の配列を基に数種のプローブを作成し、これとのハイブリダイゼーションにより行う。
【0015】また、候補遺伝子の翻訳領域を単離するために、5' RACE PCRを行ってもよい。具体的には、[2] で決定した候補遺伝子の配列を基に逆転写用のプライマーを作製し、特異的なcDNAを合成する。続いてcDNAの5' 末端にアンカーを結合させ、そのアンカーに相補的なアンカープライマーと、逆転写プライマーと重ならない候補遺伝子特異的なプライマーとの間でPCRを行う。1500-2000bp のDNA断片を単離、クローニングし、3'末端の配列が候補遺伝子と一致することを確認した上で5'末端の塩基配列を決定する。

【0016】[4] 目的遺伝子の選抜候補遺伝子の中から目的の遺伝子を選抜するために、[3]で単離した遺伝子断片を、PCR 又は制限酵素処理により翻訳領域を取り出し、大腸菌発現ベクター系にクローニングし、候補遺伝子の産物を大腸菌で発現させる。その後、大腸菌を破砕し、得られた可溶性画分を試料としてグルコース転移酵素活性の有無を確認する。活性の測定はフラボノイドの3位及び5位に対する2種のグルコース転移酵素活性を測定し、活性が検出できれば大腸菌に導入したcDNAが目的とする遺伝子であると判断する。本発明において塩基配列の決定は、ジデオキシ法 [Sanger. F, Science, 214,1205-1210 (1981)]等により行うことができる。
【0017】[5] 宿主細胞への遺伝子導入および発現上記で得られたUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子（以下、本発明遺伝子）は、適当な宿主細胞中に導入して高発現させることができる。具体的には、ベクターDNAの適当な制限酵素部位に本発明遺伝子を含むDNAを挿入して組み換え体DNAを調製し、これを宿主細胞中に導入する。宿主細胞としては、目的とする遺伝子を発現できるものであればよく、真核細胞及び原核細胞のいずれをも用いることができる。真核細胞としては動物、植物、酵母等の細胞が、また原核細胞としては大腸菌、枯草菌、放線菌等が挙げられる。
【0018】本発明遺伝子を組み込むベクターDNAは、宿主細胞で複製可能なものであれば如何なるものでもよく、例えば、プラスミドDNA、バクテリオファージDNA等が挙げられる。宿主細胞が大腸菌である場合のベクターDNAとしては、例えばプラスミドpUC18／pUC19、pKK223－3、pGEX－2T、pGEX－3X、pRIT2（Pharmacia 社製）；pGEMEX－1、pGEMEX－2（Promega 社製）；pMAL－c, pMAL－p（New England Biolabs 社製), pET15b(Novagen 社製) 等を用いることができる。
【0019】組み換え体DNAを宿主細胞を形質転換するには、Hanahan 法 ["Molecular Cloning, A Laboratory Manual", 2nd Ed., Cold Spring Harbor Laboratory Press, New York (1989)]、Chung らの方法 [Proc. Natl. Acad. Sci., U.S.A., 86, 2172 (1989)] 等を用いて行うことができる。上記のようにして得られた形質転換体の培養は、通常の形質転換体によるポリペプチドの生産に用いる培養方法に従って行われる。
【0020】大腸菌や酵母菌等の微生物を宿主細胞として用いた形質転換体を培養する培地は、微生物が資化し得る炭素源、窒素源、無機塩類等を含有し、形質転換体の培養を効率的に行える培地であれば天然培地、合成培地のいずれでもよい。炭素源としては、それぞれの微生物が資化し得るものであればよく、グルコース、フラクトース、スクロース、これらを含有する糖蜜、デンプンあるいはデンプン加水分解物等の炭水化物、酢酸、プロピオン酸等の有機酸、エタノール、プロパノールなどのアルコール類が用いられる。
【0021】窒素源としては、アンモニア、塩化アンモニウム、硫酸アンモニウム、酢酸アンモニウム、りん酸アンモニウム、等の各種無機酸や有機酸のアンモニウム塩、その他含窒素化合物、並びに、ペプトン、肉エキス、酵母エキス、コーンスチープリカー、カゼイン加水分解物、大豆粕および大豆粕加水分解物、各種発酵菌体およびその消化物等が用いられる。 無機物としては、りん酸第一カリウム、りん酸第二カリウム、りん酸マグネシウム、硫酸マグネシウム、塩化ナトリウム、硫酸第一鉄、硫酸マンガン、硫酸銅、炭酸カルシウム等が用いられる。
【0022】培養は、振盪培養または深部通気撹拌培養などの好気的条件下で行う。培養温度は通常10～50℃、好ましくは30～40℃がよく、培養時間は、通常1～10時間、好ましくは2～5時間である。培養中pHは、通常7～8に保持する。pHの調整は、無機あるいは有機の酸、アルカリ溶液、尿素、炭酸カルシウム、アンモニアなどを用いて行う。
【0023】培養物から目的酵素の単離精製は公知の分離操作を組み合わせて行うことができる。例えば、尿素などの変性剤や界面活性剤による処理、超音波処理、酵素消化、塩析や溶媒沈澱法、透析、遠心分離、限外濾過、ゲル濾過、SDS－PAGE、等電点電気泳動、イオン交換クロマトグラフィー、疎水性クロマトグラフィー、アフィニティークロマトグラフィー、逆相クロマトグラフィーなどが挙げられる。以下、本発明を実施例により具体的に説明するが、これらにより本発明の範囲が限定されるものではない。
【0024】
【実施例】
〔実施例1〕
(1) リンドウの花弁からの総RNA抽出リンドウ（品種：極晩）の花弁20gを液体窒素存在下で乳棒乳鉢を用いて粉砕した。これにグアニジンイソチオシアネート溶液（25 mM クエン酸ナトリウム、0.0025% N －ラウロイルザルコシン酸ナトリウム、0.5 mM 2 －メルカプトエタノール、4M グアニジンチオシアネート）50mlを加え、室温で溶かしながら撹拌した。続いて、フェノール抽出、エタノール

沈殿、LiCl沈殿により精製し、約5.5mgの精製全RNAを得た。次にこれをOligo(dT)-Latexを用い、既知の手法〔実験医学Vol.7, No.17, 2065-2068 (1989)〕により、ポリA(ポリA+)鎖画分を高度に濃縮した。

【0025】(2) ,,糖転移酵素遺伝子部分配列の増幅(2-1) 逆転写反応3'RACE SYSTEM (GIBCO BRL社製) を用いて行った。mRNA溶液130 ,,l(10,,g mRNA) に10,,M のadapter primer(GIBCO BRL 社製) 溶液 10 ,,l を加え、65℃10分間インキュベートし、氷上に2 分間放置した。これに10 × synthesis buffer 20 ,,l, 10mM dNTP mix 10,,l, 0.1M DTT 20 ,,l を加え、42℃で2 分間インキュベートした。次に、SuperScript RT 10 ,,l を加え42℃で30分間反応を行った。その後、氷上にサンプルを移し、10,,l のRNase H を加え42℃で10分間インキュベートを行った。

【0026】(2-2) PCR反応(2-1) の逆転写反応液1,,l に、1.25unit AmpriTaq DNA Polymerase (PERKINELMER CETUS社製) 、10 × PCR buffer (TAKARA社製) 5 ,,l 、10mM dNTP mix 4,,l 、10,,M universal amplification primer(GIBCO BRL社製)1,,l 、20,,M の,,糖転移酵素特異的配列プライマー 2.5,,l 加え、滅菌水で総量を50,,l とし、PCR 反応液とした。これをサーマルサイクラーPJ480 を用いてPCR(PCRプログラム : 94 ℃ 7分間を1サイクル; 94 ℃ 1分間→ 42, 48, 50又は55 ℃,1分間→ 72 ℃, 1 分間を30サイクル; 72℃, 7 分間を 1サイクル) を行った。

【0027】(2-3) PCR産物のプラスミドDNAへのクローニングとシーケンス解析上記のPCR産物をInvitrogen社製TAクローニングキットを用い、pCRII ベクターに導入し、INV ,,F株にクローニングを行った。シークエンスの読み取りはDNAシークエンス装置473A(PERKIN ELMER ABI 社製) を用いて行った。また、DNAシーケンスの解析はDNASIS(日立ソフトエンジニアリング社製)とSeqEd ((PERKIN ELMER ABI社製) の両ソフトで行った。

【0028】シーケンス解析の結果得られたDNA配列は、アミノ酸配列に翻訳し、図1に示す,,糖転移酵素遺伝子のコンセンサスアミノ酸配列と比較し、明らかにこの配列に当てはまるものだけを候補遺伝子の配列とした。288 個のクローンについてDNA配列の一部をシーケンス解析した結果、53個の,,糖転移酵素遺伝子配列を持つと推測されるクローンが得られた。得られたクローンは配列より12種に分類できた。

【0029】(3) ,,糖転移酵素遺伝子の翻訳開始領域の解析(3-1) 5' RACE PCR用プライマーの作製(2)で得られた12種の候補遺伝子の3'側の配列からストップコドンを確定し、それぞれストップコドンを含む30bpの相補配列をPCRプライマーとした。また、PCRプライマーに対応する配列の、すぐ下流の30bpの相補配列を逆転写プライマーとした。

【0030】(3-2) cDNA合成5'RACE SYSTEM (GIBCO BRL社製) を用いて行った。mRNA溶液14,,l (1,,g) に2.5 ,,M の逆転写プライマー溶液 1,,l を加え、70℃10分間インキュベートし、氷上に1 分間放置した。これに10 × synthesis buffer 2.5,,l, 10mM dNTP mix 1,,l, 0.1M DTT 2.5 ,,l 、25mM $MgCl_2$ 3,,l 、逆転写酵素 SuperScript RT 1 ,,l を加え、50℃で30分間、続いて70℃で15分インキュベートした。その後、1 ,,l のRNase H を加え55℃で10分間インキュベートした。合成したcDNAは5'RACE SYSTEM (GIBCO BRL 社製) 添付のカートリッジカラムを用い、添付のプロトコールにより精製した。

【0031】(3-3) 翻訳開始領域を含むDNA断片の増幅(3-2) で得たcDNAにアンカーDNA (CLONTECH社製) をT4 Ligase を用いて常法により結合させた。アンカーDNAを結合させたcDNAをGENE Amp PCR System9600 (PERKIN ELMER社製) により、アンカープライマー (CLONTECH 社製) 、(3-1) で設計したPCRプライマーを用いてPCR(PCRプログラム: 94 ℃, 45秒間→55～65℃, 45秒間→ 72 ℃, 2 分間を35サイクル) を行った。

【0032】(3-4) 5' RACE PCR産物のプラスミドDNAへのクローニングとシーケンス解析上記の増幅産物について、(2-3) と同様にクローニングを行った。シークエンスは、(2-3) と同様な装置を用いて行い、12候補遺伝子の翻訳開始領域の塩基配列を決定した。

【0033】(4) 候補遺伝子の翻訳領域DNA断片の単離(4-1) PCR プライマーの作製12候補遺伝子のアミノ酸配列のN末端の2～9番目のアミノ酸残基に相当する24塩基(または23塩基)の5' 端にベクターに組み込む為の制限酵素認識配列と、さらにその5' 端にGCGの3塩基を付けた配列を片側プライマーとした。もう一方のプライマーは、候補遺伝子の3' 非翻訳領域の相補配列において、上記のプライマーとは相補構造を形成しない配列を有する24塩基(または23塩基)の5' 端にベクターに組み込むための制限酵素認識配列と、さらにその5' 端にGCGの3塩基を付加した配列とした。

【0034】(4-2) RT-PCR による候補遺伝子ORF 領域DNA断片の作製2.5mM dNTP mix 1.6,,l 、20 ,,M PCR 両プライマー各2.0 ,,l を混合し、滅菌水で総量を10,,l とし、Ampli Wax PCR Gems 100

(PERKIN ELMER社製) を加え、サーマルサイクラーPJ9600(PERKIN ELMER 社製) で80℃、10分さらに25℃、5 分インキュベートした。これに、10×UlTma buffer(PERKIN ELMER 社製) 10„l 、25mM $MgCl_2$ 6„l 、UlTma DNA Polymerase (PERKIN ELMER社製) 1 „l 、(2-1)で作製した逆転写産物溶液1 „l 、滅菌水72„l 加え、サーマルサイクラーPJ9600(PERKIN ELMER 社製) を用い、PCR(PCRプログラム: 96℃, 1 分間を 1サイクル;95 ℃, 1 分間→55℃, 1 分間→ 72 ℃, 1 分間を35サイクル;72 ℃,7 分間を1 サイクル) を行った。

【0035】反応液は、フェノール・クロロホルム処理とエタノール沈殿処理を行い、各々のプライマーに適した制限酵素で処理し、フェノール・クロロホルム処理とエタノール沈殿処理を行った。次に、1% Seakem GTG Agarose (FCM社製) で電気泳動し、検出されたDNAバンドを常法に従い回収し、発現ベクターに組み込むDNA断片とした。

【0036】(5) 大腸菌発現系を用いた目的遺伝子のスクリーニング(5-1) 候補遺伝子ORF 領域の発現ベクターpET-15b への組み込み上記で作製した候補遺伝子ORF 領域のDNA断片を、Ligation Kit (TAKARA社製) を用い、発現ベクターpET15bに組み込み、大腸菌JM109 株にクローニングした。組み換え体の確認は、DNAシークンスにより行った。

【0037】(5-2) 候補遺伝子発現ベクターのタンパク質発現宿主大腸菌BL21(DE3) 株への導入上記で作製した候補遺伝子発現プラスミドDNAを単離し、大腸菌BL21(DE3)株に形質転換した。

【0038】(5-3) 糖転移活性検出による目的遺伝子のスクリーニング形質転換した大腸菌をLB培地でO.D.=0.6まで37℃で振とう培養後、IPTG (イソプロピルチオー„ーガラクシド, 終濃度1mM)を加え、37℃でさらに3 時間振とう培養した。集菌後、菌体ペレットをバッファー [50mM Pipes-K (pH7.0)] 1ml に懸濁し、20% Triton X-100 5„l 、10mg/ml Lysozyme 1„l を加えて30℃で15分間放置した後、凍結(-80℃) 、融解を3 回繰り返した。続いて超音波処理を懸濁液が透明によるまで行い、マイクロ遠心機で15000rpm, 15分, 4 ℃で遠心し、上清の可溶性画分を得た。

【0039】続いて、可溶性画分を脱塩カラムとしてHi-Trap desalting (ファルマシア社製)を用い、バッファー [50mM Pipes-K (pH7.0), 2% Triton X-100, 0.2mM DTT, 1mM PMSF, 50 „g/ml leupeptin, 0.54TIU aprotinin] 20ml をカラムの前処理として5ml/min.で流し、続いて該画分を1ml 流すことによって脱塩した。さらに3ml のバッファーを流し、800 „l ずつ分画し、2 番目の流出画分を活性検出に用いた。

【0040】上記の脱塩を行った可溶性画分40„l に、925 kBq/„l の[^{14}C]-UDPG 5„l 、基質色素溶液 [Del(Delphinidin), Del-3G(Delphinidin 3-O-glucoside), Cya(Cyanidin), Cya-3G(Cyanidin 3-O-glucoside)を各々溶解した5mM塩酸、基質色素なしのコントロールには5mM 塩酸] 5 „l を加え、30℃で30分間インキュベートし、クロロホルム:5% 塩酸／メタノール=2:1を50„l を加えて水層を分取した。

【0041】水層を逆相カラム・„Bondapak C18 を用いたHPLCで分離し、280nm の吸光度をモニターした。さらにフラクションコレクターで30秒間づつ分取したサンプルに液体シンチレータ2.5ml を加え液体シンチレーションカウンターLS6000TA(ベックマン社製)で各分画の放射能を測定した。インサートなしのpET15bを含むクローンの可溶性画分では基質色素への糖転移反応が起こらないことを確認したのち、候補遺伝子を含むクローンの糖転移活性を調べたところ、候補遺伝子のうちの一つのクローンで4種の基質への糖転移反応が認められた。基質別の反応結果を図2に示す。

【0042】(6) cDNA ライブラリーからの目的遺伝子cDNAの単離(6-1) リンドウ花弁cDNAの作製試料となるmRNAは、リンドウ花弁よりQuick Pep (ファルマシア社製)を用い精製した。cDNA合成はmRNAを1 „g を用い、SuperScript Plasmid System for cDNA Synthesis (BRL社製) で行った。

【0043】(6-2) cDNA ライブラリーの作製上記で作製したcDNAを、1% SeaKem GTG Agarose (FMC社製) で電気泳動し、約1.3 ～2.5kbpの長さの領域を泳動ゲルから回収し、SUPREC-01 (TAKARA 社製) で精製した。このDNA をLigation Express (CLONTECH社製) でプラスミドpSPORT1にライゲーションし、ElectroMAX DH10Bコンピテントセル(GIBCO BRL社製) にGene Pulser (Bio Rad社製) を用いて形質転換した。

【0044】(6-3) 目的遺伝子cDNAのスクリーニングGENETRAPER cDNA Positive Selection System (GIBCO BRL社製) を用いて行った。得られたcDNAクローンの確認は、DNAシーケンスにより行った。プローブは5'-ATGAAGAAAGCAGAGTTGGTTATCA-3' を、複製プライマーは5'-CATTTCCAGGGATTAGCCATGTTGG-3' の配列をもつ合成DNAを用いた。

【0045】(6-4) 候補遺伝子cDNAの配列決定上記で得られたクローンは一部分のDNA配列を決定し、目的遺伝子の配列に一致するクローンであることを確認した上、全塩基配列を決定した(配列番号1)。またこれより予想されるアミノ酸配列を配列番号2に示す。

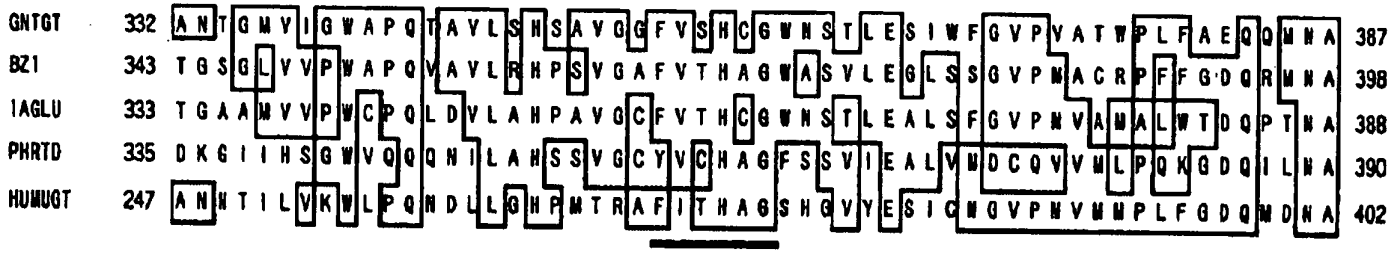
【0046】
【配列表】
配列番号：1配列の長さ：473配列の型：アミノ酸トポロジー：直鎖状配列の種類：ペプチド配列Met Lys Lys Ala Glu Leu Val Ile Ile Pro Phe Pro Gly Ile Ser His 5 10 15 Leu Gly Ser Thr Val Glu Leu Ala Lys Leu Leu Ala Glu Arg Asn Glu 20 25 30 His Leu Ser Ile Ser Val Ile Ile Ile Lys Phe Pro Asn Asp Thr Lys 35 40 45 Val Ser Asn Leu Leu Lys Ser Leu Ser Thr Ala Ser Arg Ile Lys Val 50 55 60 Ile Glu Leu Lys Gln Glu Thr Ile Ser Ile Glu Thr Gly Pro Leu Phe 65 70 75 80 Ile Gln Lys Phe Ile Glu Ser His Lys Ala Gln Val Arg Asp Phe Leu 85 90 95 Ala Gly Val Ser Ala Cys Glu Ser Val Glu Leu Ser Gly Val Val Ile 100 105 110 Asp Met Phe Cys Thr Ser Met Ile Asp Val Ala Asn Glu Phe Glu Val 115 120 125 Pro Ser Tyr Val Phe Phe Thr Ser Ser Ala Ala Met Leu Gly Leu Trp 130 135 140 Phe His Phe Gln Ser Leu Arg Asp Asn Phe Gly Lys Tyr Val Asp Ile 145 150 155 160 Lys Asp Ser Glu Thr Val Leu Ser Ile Pro Ala Phe Gln Asn Leu Val 165 170 175 Pro Tyr Gly Val Leu Pro Met Phe Ile Phe Asn Thr Glu Asp Gly Cys 180 185 190 Asp Ala Ser Leu Asp Ile Gly Lys Arg Phe Arg Glu Thr Lys Gly Ile 195 200 205 Ile Ile Asn Thr Phe Leu Glu Leu Glu Ser His Ala Ile Glu Ser Leu 210 215 220 Ser Thr Asp Glu Thr Ile Pro Pro Val Tyr Thr Val Gly Pro Ile Leu 225 230 235 240 Gly Pro Lys Gly Ser Ser Ile Glu Ser Leu Glu Thr Glu Lys Ile Leu 245 250 255 Lys Trp Leu Asp Met Gln Pro Glu Lys Ser Val Val Phe Leu Cys Phe 260 265 270 Gly Ser Leu Gly His Phe Gly Glu Ala Gln Val Lys Glu Ile Ala Tyr 275 280 285 Ala Leu Glu Gly Ser Gly His Arg Phe Leu Trp Ser Leu Arg Lys Pro 290 295 300 Pro Pro Leu Gly Lys Phe Glu Gly Pro Gly Glu Tyr Glu Asn Leu Glu 305 310 315 320 Glu Val Leu Pro Glu Gly Phe Leu Glu Arg Thr Ala Asn Thr Gly Met 325 330 335 Val Ile Gly Trp Ala Pro Gln Thr Ala Val Leu Ser His Ser Ala Val 340 345 350 Gly Gly Phe Val Ser His Cys Gly Trp Asn Ser Thr Leu Glu Ser Ile 355 360 365 Trp Phe Gly Val Pro Val Ala Thr Trp Pro Leu Phe Ala Glu Gln Gln 370 375 380 Met Asn Ala Phe Glu Leu Val Lys Glu Leu Gly Leu Ala Val Glu Val 385 390 395 400 Lys Met Asp Tyr Lys Lys Asp Tyr Lys Asn Pro Asp Ala Asp Glu Ile 405 410 415 Val Arg Ala Asp Val Ile Glu Glu Lys Ile Lys Ile Leu Met Asp Pro 420 425 430 Glu Asn Gly Ile Arg Lys Lys Val Lys Glu Met Lys Glu Lys Ser Arg 435 440 445 Leu Ala Val Glu Glu Gly Gly Ser Ser Ser Ala Ser Leu Lys Asp Phe 450 455 465 Ile Asn Asp Val Ile Lys Arg Leu Pro 470 【0047】配列番号：2配列の長さ：1597配列の型：核酸鎖の数：二本鎖トポロジー：直鎖状配列の種類：cDNA to mRNA起源 生物名：リンドウ細胞の種類：花弁配列の特徴特徴を表す記号：CDS存在位置：39..1460特徴を決定した方法：EAAGAAAGACA AAAAATTGAG
CAAAAAGAAG GGGAAAAAAT GAAGAAAGCA GAGTTGGTTA 60TCATCCCATT
TCCAGGGATT AGCCATCTTG GTTCAACAGT TGAATTAGCT AAGCTTCTTG
120CAGAGAGAAA TGAACATCTC TCCATTTCAG TCATCATCAT CAAGTTCCCA
AATGATACAA 180AGGTTTCCAA CCTCTTAAAA TCCCTATCCA CAGCTTCACG
GATAAAGGTC ATTGAGCTTA 240AACAAGAAAC CATTTCAATT GAAACCGGCC
CACTTTTTAT ACAAAAATTC ATCGAATCCC 300ATAAGGCCCA GGTGAGAGAT
TTTCTTGCTG GAGTTTCTGC TTGTGAATCT GTTGAACTCT 360CCGGGGTTGT
GATAGATATG TTCTGTACCT CCATGATTGA TGTTGCCAAT GAGTTTGAGG
420TTCCAAGCTA TGTGTTTTTC ACATCTAGTG CTGCAATGCT TGGTCTTTGG
TTCCATTTTC 480AAAGCCTTAG GGACAATTTC GGTAAATATG TAGATATTAA
AGATTCTGAG ACTGTCTTAA 540GCATCCCTGC TTTCCAAAAT CTTGTTCCTT
ATGGAGTATT GCCTATGTTT ATATTCAATA 600CGGAAGATGG ATGTGATGCA
TCTTTAGATA TAGGTAAAAG ATTTAGGGAG ACAAAAGGGA 660TCATAATCAA
TACATTTCTT GAGCTTGAAT CCCATGCTAT TGAATCTTTG TCCACAGATG
720AAACCATCCC ACCGGTTTAC ACTGTAGGGC CAATATTGGG ACCAAAAGGC
AGCAGTATCG 780AAAGCCTGGA AACCGAGAAG ATTTTGAAAT GGCTTGATAT
GCAGCCTGAG AAGTCTGTAG 840TTTTCCTTTG TTTTGGTAGC CTTGGTCATT
TTGGTGAGGC CCAAGTGAAG GAAATCGCAT 900ATGCACTAGA GGGTAGTGGC
CATAGATTCT TGTGGTCATT AAGAAAGCCA CCACCTTTAG 960GGAAATTTGA
AGGCCCTGGT GAGTATGAGA ACTTGGAAGA GGTCCTCCCA GAAGGGTTTT
1020TAGAACGCAC AGCTAACACC GGAATGGTGA TTGGATGGGC CCCACAGACG
GCCGTGTTGT 1080CTCACTCAGC TGTGGGAGGT TTCGTGTCGC ATTGTGGATG
GAATTCAACA CTGGAAAGCA 1140TTTGGTTTGG TGTCCCAGTG GCAACCTGGC
CTCTGTTTGC TGAGCAGCAG ATGAATGCAT 1200TTGAATTGGT GAAGGAATTG

GGCTTGGCTG TGGAGGTTAA GATGGATTAT AAAAAAGATT 1260ACAAGAATCC
TGACGCGGAC GAAATAGTGA GAGCTGATGT TATAGAAGAG AAAATCAAGA
1320TATTGATGGA TCCTGAAAAT GGAATCAGAA AGAAAGTGAA GGAAATGAAA
GAAAAGAGCA 1380GGCTGGCTGT TGAGGAAGGA GGATCATCTT CTGCGTCCCT
CAAAGATTTT ATTAATGATG 1440TGATCAAAAG ACTTCCATGA TTTAGCTCTT
CCAAATTGGT TCAATAGATT TTATGGGCAA 1500AACTGTTAAG TTGCTCTTTG
CATTAGAGCA AATGCTCTGA TTTAATGTAA GTTTTTATTT 1560TTATTTTTTA
TTTATCTGAA ATTAAGTTTA CAAAAAA 1597【0048】
【発明の効果】本発明によれば、リンドウの花弁色素であるゲンチオデルフィンの糖鎖構造の生合成に関与する酵素遺伝子であるUDP－グルコース：フラボノイド3，5－O－グルコシルトランスフェラーゼ遺伝子が提供される。本発明の遺伝子を公知の方法に従い植物体に導入すれば、花色の色彩の調節、特にバラ等の青色花弁をもたない品種においてゲンチオデルフィンを発現させることによる青色花弁の創出が期待できる。

【図面の簡単な説明】
【図1】各種,,糖転移酵素遺伝子のアミノ酸配列の比較を示す。
【図2】糖転移酵素活性の認められた1クローンの基質別 [A. Del(Delphinidin), B. Del-3G (Delphinidin 3-O-glucoside), C. Cya(Cyanidin), D. Cya-3G(Cyanidin 3-O-glucoside)]の反応結果を示す。

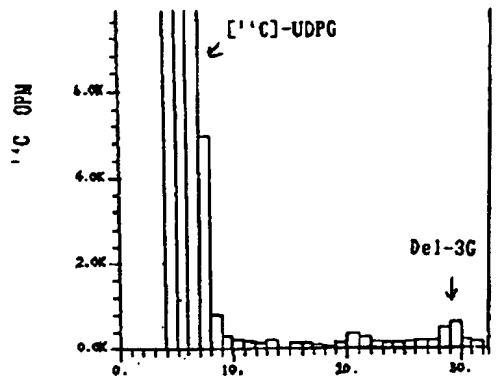
【図1】

【図2】　β糖転移酵素遺伝子のアミノ酸配列比較

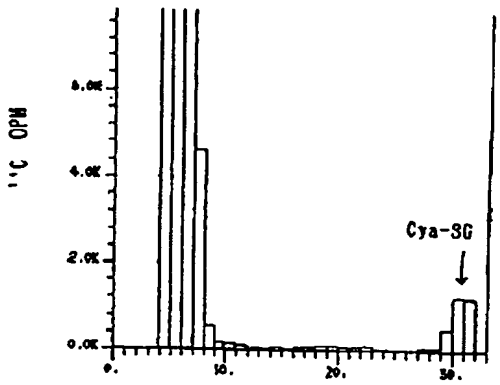


GNTGT：ﾘﾝﾄﾞｳUFGT　BZ1：ﾄｳﾓﾛｺｼUFGT［Ralston E.J. et al. GENETICS 119:185-197(1988)］　1AGLU：ﾄｳﾓﾛｺｼiaglu［Szerszen B.S. et al. Science 265:1699-1701(1994)］ PHRTD：ﾍﾟﾁｭﾆｱﾗﾑﾉｰｽ転移酵素［Kroon J. et al. Plant J. 5 (1), 69-80 (1994)］　HUMUGT：ﾋﾄUDP-ｸﾞﾙｸﾛﾝ酸転移酵素［Ritter J.K.et al. J. Biol. Chem. 267:3257-3261(1992)］

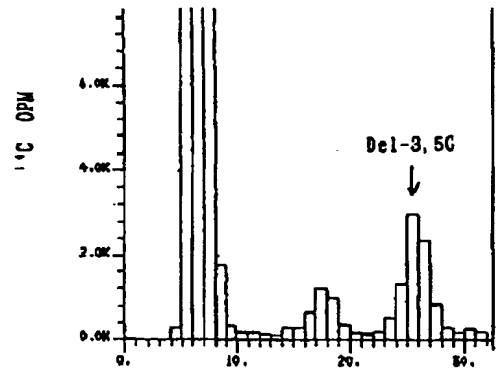
A. 基質：Del



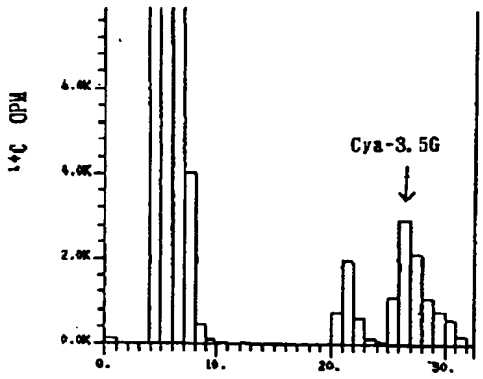
C. 基質：Cya



B. 基質：Del-3G



D. 基質：Cya-3G



分析条件

装置:SYSTEM GOLD（ベックマン社製）

カラム: μBondapak C18 3.9 ×300mm (Waters)

流速 : 1.5ml/min

移動相 A : 4.5%ギ酸

B : アセトニトリル

グラジエント :

時間（分）		（%）
5	B. CONC	3
20	B. CONC	22
22	B. CONC	50
24	B. CONC	3
35	B. CONC	3